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STUDIES ON TYPHUS AND SPOTTED FEVER

ANNUAL PROGRESS REPORT

by

Charles L. Wisseman, Jr., M.D.

February 1979
(For the period 1 July 1977 to 30 June 1978)

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Department of Microbiology ✓
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Rickettsial preparations were enriched for viability by density gradient centrifugation. The genome size of typhus group rickettsiae, Rochalimaea quintana, Baker's vole agent and R. rickettsii was found to be about 1×10^9 daltons. Hybridization techniques verified clusters of strains of putative R. prowazekii to be very closely related and the same with strains of R. mooseri but that the two groups were clearly different from each other and from R. rickettsii and Rochalimaea quintana. Baker's vole agent, suggested by others to be a strain of R. quintana, was found to be distinct by DNA homology and SDS-PAGE profiles of

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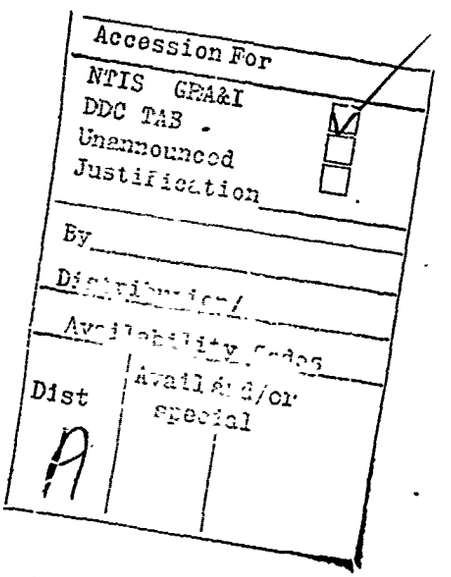
proteins. A combination of iso-electric focussing and slab SDS-PAGE revealed at least 100 protein bands in R. prowazekii. Stock-piles of antigens, specific mouse antisera and materials for DNA homology are being accumulated for definitive studies with established members of the spotted fever group and with field isolates from Pakistan, Thailand, Czechoslovakia and Israel. Lymphocyte blast transformation with lymphocytes from immune human subjects (lab personnel) on stimulation with R. prowazekii antigens was confirmed. A lymphocyte product which appears to be specifically cytotoxic to R. prowazekii-infected human fibroblasts was discovered.



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CONTENTS

I. STUDIES ON TYPHUS

A. Studies on typhus DNA and genetics. Because of certain unexpected results with the action of stimulated lymphocyte products on R. prowazekii-infected cells (see Section B-2 below), which perhaps represent a new type of mediation of typhus immunity and which consumed an enormous amount of personnel time and effort, only certain of the goals of the study of the genome of R. prowazekii were realized - viz., (1) enrichment of R. prowazekii suspensions for viability (and possibly for competence) and (2) completion of studies on genome size and homology in the typhus group, with observations on R. rickettsii, Rochalinaea quintana and Baker's vole agent. There is the possibility that some of the active lymphocyte products, if produced by other cells as interferon is, may give a clue to the nature of the vexing problem which we encountered earlier of the limitation of detection of mutant frequencies of less than 10^{-4} or 10^{-5} by the plaque technique because of either suppression of plaque formation or lysis of the cell sheet.

1. Enrichment of purified R. prowazekii suspensions for viability and other properties (L and H bands) and storage stability of purified suspensions (with Barbara Hanson). One of the first applications of Renografin gradients that we have encountered was the selective enrichment of Bacillus subtilis populations from cultures for cells in the competent state for transformation studies (1). Subsequently, Weiss and his group (2) used Renograffin gradients for the purification of rickettsiae from host cell components, a procedure which we, as many others, have adopted as a routine procedure. However, as with certain other gradient procedures, we frequently noticed two bands of rickettsiae, and sometimes some rickettsiae of intermediate density. The possibility existed that the two bands represent populations of R. prowazekii with significant differences in properties that might be of importance in the genetic and DNA studies, as well as many other studies on rickettsiae. Accordingly, rickettsiae in these two bands, derived from infected yolk sacs, were subjected to a series of tests and analyses. Not knowing in advance what the differences might be, we chose two standards of reference for comparison: (1) total RLB particle count and (2) protein, as determined by the Lowry method.

a. Density differences between light (L) and heavy (H) bands. A typical Renografin gradient of R. prowazekii harvested from live yolk sacs produced and harvested by conventional techniques usually revealed a large light (L) band with a peak at a density of about 1.13 and a smaller heavy (H) band with a peak at a density of about 1.15 (see Figure 1). The detection of two bands was a regular phenomenon, although the proportions of L and H band would vary somewhat. A marked increase in H band over L band was detected in a lot of frozen infected yolk sac that had gone through a freezer breakdown, suggesting that the H band might consist of damaged rickettsiae.

b. Morphology and identity of organisms in L and H bands. Conventional Gimenez-stained smears of L and H bands revealed that both bands consisted of a mixture of small cocco-bacillary to bacillary to bipolar organisms compatible with rickettsiae. Indirect immunofluorescence revealed that organisms in both bands reacted with anti-typhus serum. (Since the strains of R. prowazekii employed here had been plaque purified, seeds prepared in specific pathogen-free eggs and devoid of the confusing extraneous bacteria often encountered in lines passed for long periods in conventional eggs and produced by single passage in conventional eggs, it is unlikely that

the preparations contained significant numbers of extraneous egg origin bacteria. However, it was necessary to prove that both bands were typhus rickettsiae).

Electron microscopy of ultrathin sections of organisms from L and H bands, however, revealed substantial differences in morphology. L band organisms tended to have wavy outer membranes closely apposed to the inner membrane. The cytoplasm stained so densely that individual ribosomes or DNA strands was virtually impossible. In contrast, H band organisms appeared swollen, tended to have smooth outer membranes not necessarily closely apposed to the inner cytoplasmic membrane and a less dense cytoplasm in which ribosomes (somewhat fuzzy in appearance) and DNA strands were easily discerned.

c. Infectivity of L and H bands. By plaque assay in CE cell monolayers, the H band was found to have greatly reduced infectivity when compared with the L band. Indeed, in a series of different preparations, the number of plaques in the H bands varied between 0.003% and 1.17% of the number in the L bands when expressed as PFU/mg protein. The number of rickettsial bodies (RLB) per PFU was between about 40 and 7000 times greater in the H bands as compared with the corresponding L bands. In standard (in our laboratory) dose-response uptake experiments, it was found that H band rickettsiae had lost their capacity to attach to, and penetrate into, CE cells. Thus, the L band appears to be enriched in viable organisms whereas the H band consists primarily of non-infectious organisms.

d. Metabolic capacity of L and H bands. On a per organism (RLB) basis, the H band rickettsiae had greatly decreased capacity to oxidize glutamate as measured by the release of $^{14}CO_2$, the activity of the H band organisms being only 2-5% of t'at of L band organisms.

e. Permeability of L and H band rickettsiae. When examined by either phase contrast microscopic or electron microscopic methods, L band organisms were plasmolyzed by hypertonic sucrose and Renografin solutions whereas H band organisms were not, indicating that the L band organisms retain a permeability barrier to solutes whereas the H band organisms do not. Moreover, penetration of Renografin into H band organisms and its exclusion by L band organisms may contribute to the difference in densities of the two populations.

f. Macromolecule content of L and H band rickettsiae. The difference between L and H band organisms in protein, RNA and DNA content on a per RLB basis was not as great as might have been expected between viable and non-viable organisms and did not form a reproducible pattern. The H/L ratio for total protein (Lowry) varied between 0.83 and 1.11; for RNA, between 0.81 and 1.14; and for DNA, between 0.64 and 1.05. In 3 separate experiments, the RNA/DNA for L bands varied between 2.5:1 and 4.4:1 and for H bands, between 2.7:1 and 5.5:1. The latter is especially surprising in view of the reported loss of RNA by typhus rickettsiae under unstable conditions (3). Moreover, a protein profile of L and H band rickettsiae by PAGE techniques (by Oaks and Smith) failed to reveal significant differences (Figure 6).

We and others have observed that purified rickettsiae were inactivated by short storage at low temperatures. In fact, some rickettsiologists have found it necessary to use only freshly purified, unfrozen organisms in their studies. The disadvantages of this are obvious, from the standpoints both of efficiency and of reproducibility of experiments with a standard preparation. Therefore, we undertook to determine what conditions would allow the maintenance of rickettsial integrity during freezing, thawing, and storage at -70°C.

Yolk sac suspensions of R. prowazekii were purified by centrifugation through 50% sucrose, further removal of host components with anti-yolk sac antibody, and sedimentation through Renografin density gradients. To see how well these highly purified organisms could withstand rapid freezing and thawing, they were suspended in a variety of diluents and plaque-assayed before and after one or two freeze/thaw cycles. We have found several diluents in which rapid freezing and thawing caused no detectable loss of rickettsial infectivity. Accordingly, the longterm survival of typhus organisms at -70°C in these and other diluents also was tested. We have determined that highly purified R. prowazekii can survive at least 100 days at -70°C in a variety of suspending media without a noticeable decrease in viability. While factors such as NAD and glycerol had a profound protective effect under the adverse conditions of a freezer breakdown, under normal, optimum conditions the rickettsiae survived in very simple diluents.

These findings have great practical value. They permit us to produce large batches of highly purified rickettsiae (1) which are substantially enriched in organisms with high viability and metabolic activity (and hopefully in competence for transformation) by selectively harvesting L band organisms or (2) which yield the maximum amounts of certain macromolecular components, such as protein and nucleic acids, by collecting both L and H bands. Moreover, these findings have allowed us to store large batches of highly purified rickettsiae for use in a whole series of experiments. Thus, we have available preparations which have already been characterized in terms of particle numbers, plaque forming units, and Renografin gradient profiles, enabling us to standardize conditions within and between experiments.

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2. Studies on rickettsial DNA (with W.F. Myers). During the past few months the studies on rickettsial DNA have concentrated upon refining methods of extraction and purification of rickettsial DNA, shearing to standard length fragments for re-association methods, and optical methods for determining melting points (Tm) for calculating % G+C and for re-association kinetics for the determination of genome molecular weight and degree of homology between strains. Considerable progress has been made with established and recently isolated strains of the typhus group of rickettsiae and with trench fever and vole agents and a beginning has been made on the spotted fever group (see below). The results not only have basic biological and taxonomic significance, but they also are beginning to provide information of practical epidemiological significance.

Table 1. Source, passage history and other pertinent information on the strains employed in this study.

Organism/strain	Source	Passage History	Plaque-Purified	Ref.
Rickettsia prowazekii				
Breinl	Man	E155/TC3/E4	yes	
Madrid E	Man	CRD-3 /TC3/E4	yes	
Bur X-16	Man	TC5/E2	yes	
GV-F12	Flying squirrel	E7	no	
Rickettsia mooseri (*R. typhi*)				
Wilmington	Rat	E42/TC3/E3	yes	
Pak NA-18	Rat	GP/TC5/E3	yes	
Ethio AZ-306	Rat	TC5/E3	yes	
Rickettsia rickettsii (Shiela Smith)	Man	E6/PLP/E2/TC4/E6	yes	
Rochalimaea quintana				
Fuller	Man		yes	
Heliodoro	Man		no	

Table 2 GC content and genome size in strains of R. prowazekii, R. mooseri, R. quintana and E. coli.

Biotype/ strain	GC Content		DNA Prep	No. Runs	M.W. ($X10^{-7}$) (daltons ± S.D.)
	Tm	%GC			
Rickettsia prowazekii					
Breinl	81.2	29.0	1	5	106 ± 6.6
			2	16	112 ± 9.2
Madrid E	81.0	28.5	1	1	(111)
Burundi (V-16)	81.5	29.7	1	6	108 ± 6.7
Flying Squirrel (GV-F12)	81.4	29.5	1	8	114 ± 5.8
Rickettsia mooseri (typhi)					
Wilmington	81.2	29.0	1	17	109 ± 7.9
Pakistan (NA-18)	81.0	28.5	1	8	107 ± 6.1
Ethiopian (Eth-306)	81.2	29.0	1	3	107 ± 4.0
Rickettsia rickettsii					
Shiela Smith	82.4	31.9	1	7	148 ± 12
Rochalimaea quintana					
Fuller	85.4	39.3	1	4	95 ± 3.5
Heliodoro	85.6	39.8	1	3	101 ± 2.4
Baker's Vole Agent	85.3	39.1			
Escherichia coli, K-12	90.5	51.7	1	6	241 ± 13

Table 1 records some pertinent features of the rickettsial strains employed in these studies.

a. GC content and genome size of rickettsial strains. Table 2 presents an updated version of the determination of the % G+C calculated from the Tm and the molecular weight of the genome calculated from re-association kinetics. The DNA from established strains of R. prowazekii and R. mooseri as well as recent field isolates tentatively considered to be strains of either species primarily on serological grounds all possessed the expected G+C content that has become a characteristic of the typhus group. Likewise, the DNA of both the Fuller and Heliodoro strains of Rochalimaea quintana, as well as Baker's Vole Agent (1), had a similar, expected G+C content which differs substantially from the values obtained with typhus group and spotted fever group, supporting the concept that these 3 strains may belong to a single group (see below). As a beginning for the detailed study of spotted fever group organisms (see below), R. rickettsii (Shiela Smith) was included in some of these studies as well as the DNA homology studies described below.

Table 3. Determination of DNA Fragment Length on Sheared DNA

Sample	Species	Fragments measured	Mean size I.S.D.
1	R. prowazekii (Breinl)	255	$2.1 \times 10^5 \pm 1.0$
2	R. prowazekii (Breinl)	408	$2.9 \times 10^5 \pm 1.2$
3	R. mooseri (Wilmington)	206	$2.5 \times 10^5 \pm 1.1$

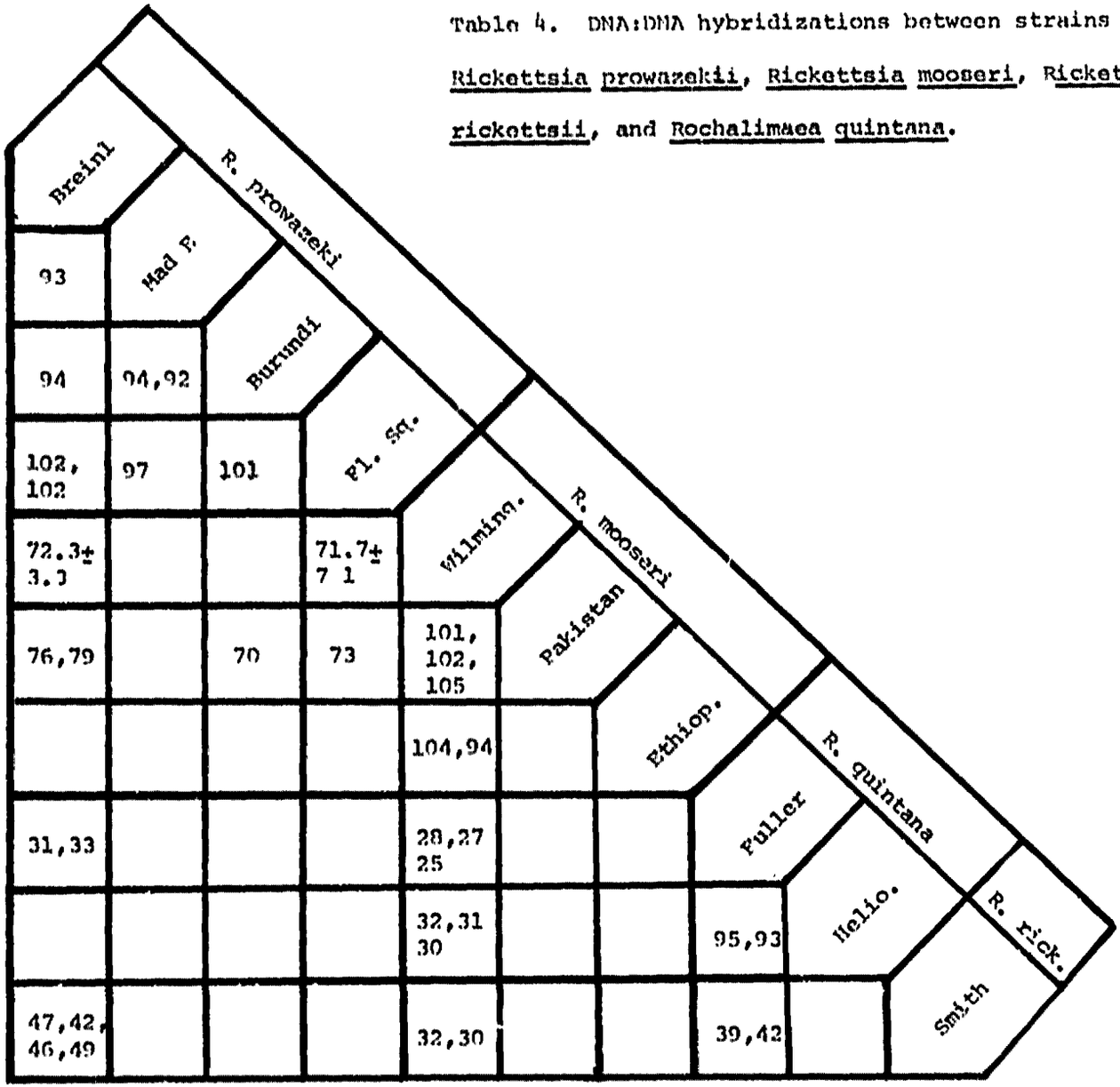
(1) Fragment length of sheared DNA. Since the determination of genome size and of DNA homology by re-association kinetics depends upon uniformity of size of fragments in the sheared DNA, accomplished in these studies by passage through a Ribi apparatus under standardized conditions, the mean fragment size of 3 typhus group DNA preparations was measured from electron micrographs of Kleinschmidt type preparations. Table 3 shows that the method produces fragments of reasonably reproducible size, suitable for re-association kinetic studies.

(2) Genome size of rickettsial strains. Table 2 shows that the genome size, calculated from re-association kinetics, of strains of R. prowazekii, R. mooseri, Rochalimaea quintana, Baker's Vole Agent and R. rickettsii all have a genome of similar size - viz., close to 1×10^9 daltons. This is a substantial genome size, about 40% the size of the genome of E. coli, about the same size as the genome of Neisseria sp. and theoretically capable of coding for about 3-400 proteins (see section on protein analysis by iso-electric focussing and SDS-PAGE).

b. (3) Homology between the DNA of rickettsial strains. Taxonomic criteria for the rickettsiae have been limited, variable and inconsistent and often unsatisfactory and have relied heavily upon such factors as light microscopy, host range, serological reactions, vector, epidemiology and geographic distribution. As a result, many important questions of taxonomic, biological and medical-epidemiological importance remain unresolved, More recently, additional characteristics, such as metabolic patterns, ultrastructure, host cell association and growth cycle characteristics, protein profiles and G & C content of DNA, have helped somewhat, especially in assigning organisms to groups - e.g., typhus group, spotted fever group, Rochalimaea, etc. They have been less helpful in differentiating species from one another. This problem is particularly severe in the spotted fever group.

On the other hand, measurement of the relatedness of the DNA's of organisms by homology techniques may provide information of an order above that which can be obtained by the other methods (7). We have made considerable progress with

Table 4. DNA:DNA hybridizations between strains of *Rickettsia prowazekii*, *Rickettsia mooseri*, *Rickettsia rickettsii*, and *Rochalimaea quintana*.



one method for measuring DNA homology among rickettsial strains which appears to be useful at the species level. It is possible that modification of the method will prove to be useful in the search for minor differences among strains of a species, but at this time sorting strains into species is the main objective. The results to date, presented below, illustrate both the taxonomic and the practical medical-epidemiological value of the information obtained by this approach.

(1) DNA homology studies with strains of the typhus group of rickettsiae, with observations on their relatedness with Rickettsia rickettsii and Rochalimaea quintana. On the basis of reassociation kinetics between denatured strands of sheared DNA measured by optical methods at a single temperature (7), established strains of Rickettsia prowazekii and Rickettsia mooseri (R. typhi) and recently isolated strains tentatively assigned to one or the other species on serological grounds were compared with respect to relatedness of their respective DNA's. Table 1 lists the strains employed, along with some basic information. Table 4 lists the results obtained, expressed as % hybridization under the conditions employed. The following conclusions can be drawn from these results:

(a) The established Breinl and E strains, a recent isolate from a patient suffering from louse-borne typhus in Burundi and a strain of rickettsia isolated from flying squirrels in the U.S. (obtained from Bozeman, et al.) all show a high degree of relatedness and probably can be considered as strains of Rickettsia prowazekii.

(b) The established Wilmington strain, a strain from a rat from Ethiopia and a strain from a rat in Pakistan all show a high degree of relatedness and probably can be considered as strains of Rickettsia mooseri (R. typhi).

(c) The relatedness between the DNA from the cluster of R. prowazekii strains and from the cluster of R. mooseri is significantly lower (ca. 70%) than among strains comprising each of the groups. This difference, along with biological, epidemiological and serological differences, is probably great enough to justify the differentiation into 2 separate species.

(d) Although the two strains of Rochalimaea quintana show a high degree of relatedness, neither the strains nor the Sheila Smith strain of Rickettsia rickettsii show any significant relatedness among themselves or to the prototype strains of R. prowazekii and R. mooseri. Indeed, the low degree of homology between typhus group organisms and R. rickettsii is consistent with other criteria; they show some degree of serological cross-reaction but do differ by a small but significant amount in the G & C content and display certain biological differences.

Thus, the taxonomic value of the DNA homology studies is beginning to emerge. Already, these have certain practical medical-epidemiological-biological applications. The following are a few examples of the practical significance of these results.

(a) Strains putatively R. prowazekii, isolated at widely separated points in time and place (3 continents - Europe, Central Africa and North America), with different degrees of passage in the laboratory, with distinct variations with respect to virulence for man (E strain), appear homogeneous by this technique. Minor differences in certain biological properties, virulence for man and certain laboratory animals and in protein profile are known, but the DNA homology results are consistent with the serological characteristics by conventional techniques (these have serious limitations) and may (remains to be proven) reflect capacity to cross-immunize.

(b) The flying squirrel agent, an epidemiological anomaly and enigma at present, nevertheless appears to be a bone fide strain of R. prowazekii, as others have suggested on the basis of serological studies. This has enormous implications. Does it mean that a strain of the organism that causes epidemic typhus in Man is actually established in a zoonotic cycle in nature and, if so, how did it get there - from Man - or is it a primordial precursor of the human line? Or is there a series of distinct and independent strains of an organism distributed in nature, including Man, which by current methods and criteria fall into a single species, but which may exist in nature as distinct organisms? Since Man and his direct antecedents are relatively recent on the evolutionary scale, does this flying squirrel agent represent a modern branch of an organism originally present in lower mammals but which may have subsequently adapted to Man, or his ancestors, which is the strain we, as medically oriented persons, have first identified and anthropocentrically oriented our concepts of microbial evolution? (See below)

(c) From time to time, it has been postulated that R. prowazekii was derived from R. mooseri by serial louse-Man passage. The significantly low homology between R. prowazekii and R. mooseri strains demonstrated in these studies strongly suggest that R. prowazekii could not have originated from R. mooseri by a single or even a small series of mutations selected by the louse-Man transmission cycle. This suggests that speciation must have occurred in the distant past, on an evolutionary scale, possibly long before Man or his immediate precursors entered the scene. This is consistent with the hypothesis that different R. mooseri-like and R. prowazekii-like strains exist in nature in lower animals (as in the case of the flying squirrel agent). The R. prowazekii that we now encounter in classical louse-borne typhus in Man may simply be the selection of one of these strains to the louse-Man cycle.

(d) Similarly, the cluster of strains that constitute R. mooseri might have been artificially selected from a series of R. mooseri-like strains in nature by the vehicle of Rattus rattus, Rattus norvegicus or both, along with Xenopsylla cheopis which constitute a fortuitous combination for the perpetuation and world-wide dissemination (through the early migrations of Rattus species, later supplemented by improved communications afforded by Man) so that what we see, when we isolate organisms from various sites of human concern, is the strain that was selected by the Rattus-flea system and disseminated by Man. There is the possibility that other R. mooseri-like strains exist in different cycles and places and

that we might find them if we look in the right places. For example, the prototype Wilmington strain, isolated in the U.S., was undoubtedly transported here by the Rattus-flea system. The ecological evidence from Ethiopia is that R. mooseri was confined to Rattus rattus and its ectoparasites and was a recent introduction. In contrast, in Rangoon, Burma, all five of the rodent species and one species of shrew intimately associated with human dwellings in large numbers are, on serological grounds, infected with "R. mooseri" (and we have recent evidence of human cases of murine typhus there). Does this mean that we are getting close to the primordial source of murine typhus and, if so, will the strains isolated from there all be identical or will they show some minor variations? Or is this a special case where a single strain of Rattus-borne R. mooseri has been introduced by the Rattus-flea system and has spilled over into other commensal small mammals because they all share a common site (houses) and share the same limited number of ectoparasite species? We hope soon to have isolates from the various commensal small mammal species from Rangoon for study.

The practical consequences of these theoretical considerations are important. For example, if a variety of R. prowazekii-like strains exist in nature, do they constitute a health hazard of significance above and beyond the classical louse-borne typhus? If so, will vaccine protective against classic louse-borne typhus also protect against these strains? Control of murine typhus through measures directed at Rattus rattus and R. norvegicus may be effective when these are the only mammalian "reservoirs" concerned. However, control of multiple species of small mammals, some insectivores and others with diverse feeding, breeding and nesting habits, is extremely complex. Will the selective destruction of one species result in an expansion of the population of an even more effective system of dissemination?

(2) Differentiation between Rochalimaea quintana strains (agent of Trench Fever) and Baker's vole agent by DNA homology. On the basis of morphological, cultural (4, 5) metabolic and cell association characteristics, strengthened by an indistinguishable % G & C in DNA and by superficial serological cross-reactions, Weiss and his associates (2, 3) concluded that Baker's vole agent (1) is a strain of Rochalimaea quintana, the agent of trench fever. Unfortunately, the serological studies were only sufficient to demonstrate some degree of cross-reaction between the two organisms but were not adequate to differentiate between two related, but different, organisms.

We have confirmed that, indeed, the % G & C, by melting point determinations, of the vole agent and two strains (Fuller and Heliodoro) of R. quintana are essentially the same. However, when these strains were compared on the basis of the degree of homology between their respective DNA's, marked differences were found between the established R. quintana strains and lines of the vole agent. Table 5 shows the following: (1) a high degree of homology between the DNA's of two lines of the Fuller strain and the Heliodoro strain of Rochalimaea quintana; (2) a high degree of homology between the DNA's of lines of the vole agent; (3) a very low degree of homology between the DNA's of strains of Rochalimaea quintana and lines of the vole agent. Furthermore, substantial differences between R. quintana and the vole agent have been found in the SDS-PAGE protein patterns (See Figure 8).

Table 5. DNA Homology Studies Between Strains of Rochalimaea quintana and Baker's Vole Agent.

Strain[1,2]	% Homology					
	Fuller 1	Fuller 2	Heliodoro	Vole 1	Vole 2A	Vole 2B
Fuller 1	100		94	28		
Fuller 2		100	97		36	
Heliodoro			100	34		24
Vole 1				100	104	96
Vole 2A					100	
Vole 2B						100

1 Strains of R. quintana

Fuller 1 : Fuller strain long in Department (pure culture)
Fuller 2 : Fuller strain recently acquired from ATCC
Heliodoro : Heliodoro strain from Departmental Collection
Vole 1 : Baker's Vole Agent from Departmental Collection
Vole 2 : Baker's Vole Agent recently acquired from ATCC
A and B are progeny of two different colonies picked from agar culture.

We can only conclude that R. quintana and Baker's vole agent are different organisms, possibly representing two distinct species of the genus Rochalimaea. Thus, the power and usefulness of DNA composition and DNA homology studies in sorting out the genera and species of organisms included in the Rickettsiales is demonstrated.

From the point of view of human health relevance and the epidemiology-ecology of trench fever, it is of considerable practical importance to know that a potentially large wild animal reservoir of the trench fever agent, R. quintana, does not exist in nature. From a biological point of view, it is of considerable interest to know that agents with similar, unique biological properties, perhaps different species of the same genus, exist in nature in animals as distantly related as Man and voles. This again points to an early evolutionary diversification of related agents with the recent establishment of a strain selected and adapted to the Man-louse cycle.

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B. Studies on immunity in typhus. In addition to our laboratory and field studies on the living attenuated E strain typhus vaccine, we have maintained a long-standing interest in the basic immune mechanisms in typhus infection which have included studies on the direct action of antibodies on typhus rickettsiae, cytophilic and opsonizing antibodies and interactions with phagocytic cells (5-19).

Although human convalescent serum did not have any direct rickettsiacidal action on typhus rickettsiae, it did opsonize them for enhanced phagocytosis by both polymorphonuclear and monocytemacrophage cells but it did not inhibit penetration and growth in non-phagocytic cells. Human macrophages phagocytized the rickettsiae but the rickettsiae quickly escaped from the phagocytic vacuoles into the cytoplasm where they grew and eventually destroyed the macrophages. Immune serum passively transferred to mice protected them against death from i.p. R. mooseri infection. In similarly adoptively immunized guinea pigs, antibodies failed to restrict rickettsial proliferation at the site of inoculation in skin but did modify systemic spread. Thus, we have defined to some extent, but not completely, the limitation and mechanisms of action of antibody-mediated immunity. [As early as 1927, Krontowski and Hach (1) observed that the growth of typhus rickettsiae was not inhibited in guinea pig tissue explant cultures, but no further in vitro work was done on this until we revived it a few years ago. A protective action of previously administered immune serum to animals was reported later by numerous investigators.]

The possible importance of cell mediated immunity in typhus, not recognized specifically as such, came from the work of Castaneda (2, 3) in 1936, who drew the analogy between the reaction to innoculation in immune guinea pig skin and the Koch phenomenon in tuberculosis. Later, skin tests which resembled delayed type hypersensitivity were used extensively for epidemiolgical, but not immunological, purposes (reviewed in 8). In early studies with the attenuated E strain vaccine in Man (8), we demonstrated that vaccinated subjects became skin-test positive (delayed type hypersentivity) 7-10 days after vaccination, often before serum antibodies were detectable and just before the "late" reaction to vaccine in those subjects who experienced such a reaction. More recently, we have begun a more systematic investigation of cell mediated immunity in typhus in Man when possible, and animals. Significant is the fact that, in the R. mooseri-guinea pig model (19), CMI as measured by skin test, was not demonstrable after a single dose of potent experimental killed vaccine; nor was it positive after R. mooseri infection. It became strongly positive when the killed vaccine was administered in complete Freund's adjuvant. However, when CMI was measured by the correlate of MIF production, it was found that a single dose of killed vaccine alone did not elicit CMI but that both killed vaccine in complete Freund's adjuvant and previous infection elicited strong CMI. A "blocking factor" was identified in the serum of infected animals which prevented the development of a positive skin test. When challenged by the intraperitoneal route with R. mooseri, animals which received killed vaccine alone did not resist challenge (as measured by fever) whereas animals which had received either killed vaccine in complete adjuvant or which had recovered from infection resisted challenge.

Further analysis (15-18) was made in the R. mooseri-guinea pig model, in which infection and immunity was measured by quantitative assay by plaque count of the growth and disappearance of rickettsiae in various tissues after intradermal inoculation of R. mooseri, much as Castaneda had done in 1936. This proved fortuitous, for an infection was established which progressed successively, on a practical time scale, from inoculation site in the skin, to draining lymph nodes, to blood and finally to distant organs such as spleen and kidney. The infection at the site of inoculation in the skin evolved and was controlled before serum antibodies were detectable. Infection of the spleen occurred after serum antibodies were present. It evolved and was controlled in 3-5 days despite presence of serum antibodies. Similarly, the infection in the kidney evolved in the face of serum antibodies, but in this apparently privileged site infection persisted for at least 28 days. This delayed expression of immunity at distant sites after control at the site of inoculation bears some similarity to the antigen - lymphocyte-dependent homing of lymphoblasts described by Jungi and McGregor (30) for Listeria. Passive transfer of large quantities of convalescent guinea pig serum to non-immune recipients failed to control the infection in the skin at the site of inoculation but passive transfer of 10^9 spleen cells from immune syngeneic guinea pigs did control the skin infection. However, both passively transferred immune serum (possibly by enhanced clearance mechanisms by phagocytic cells) and immune spleen cells modified the infection in distant organs. Although T cells were not specifically identified as the mediators of the strains immunity imparted by immune spleen cells, these studies point to the salient role of CMI in control of typhus infections. Another graduate student is currently developing an inbred mouse model in which methods for cell separation have been worked out in the attempt to define the specific lymphocyte populations responsible for conferring cell mediated immunity in typhus. Collectively, these studies suggest an important, perhaps dominant, role of CMI in typhus immunity.

Similar observations are being made by others in scrub typhus (20, 21) and Q fever (24, 26).

The studies described below began as a simple extension of the observations of Coonrod and Shepard (4) on lymphoblast transformation in human subjects with past typhus infections because several such subjects were available in the laboratory (#1 below). However, the observation that supernatants from stimulated lymphocytes seemed to have a specific effect on R. prowazekii-infected somatic human cells, as opposed to macrophages, immediately opened an exciting new area of study on the mechanisms of cell-mediated immunity to typhus which could operate on cells that constitute the primary site of infection in tissues, as opposed to the classical TB-Listeria model of CMI (28, 29) which in fact just is another way in which macrophages might be induced to control rickettsia, a mechanism that is beginning to gain some attention with scrub typhus (22, 23) and Q fever (25). The notion that we might be dealing with a new phenomenon in the control of intracellular parasites was re-inforced by the work of Chinchilla and Frenkel (27) on toxoplasma which appeared while these studies were in progress. Accordingly, we have devoted almost all of our attention to this phenomenon in the last few months, to the serious neglect of certain other projects on genetics of R. prowazekii because the available man-power was not sufficient to handle both.

1. Lymphoblast transformation in human subjects infected with typhus rickettsiae. (With Lorraine Fiset) With the mounting evidence for the importance of delayed type hypersensitivity and cell mediated immunity in typhus infections (; see above) and the availability in the laboratory of human subjects who had experienced typhus infections in the past or who had been exposed to typhus antigens under a variety of circumstances, an unique opportunity expressed itself when, at the same time, the skills of Dr. Lorraine Fiset, who had previous experience with lymphoblast transformation assay techniques, became available to us for a short period of time. Accordingly, we embarked, with due respect for the limitation of this lymphoblast transformation assay, upon a project to take advantage of the typhus immune human population at hand to expand, in one of the few ways that one can investigate information about CMI in human subjects, the limited, but significant observations of Coonrod and Shepard (4) on lymphoblast transformation as a correlate of CMI in Man.

The typhus antigens employed were purified suspension of formalin-killed R. prowazekii (Breinl). The methods employed were standard techniques of the measurement of lymphocyte blast transformation based upon the uptake of ^{3}H-thymidine. An extensive series of preliminary control and calibration experiments were performed. The endotoxic activity of suspensions of R. prowazekii were previously demonstrated in these laboratories by an extensive series of biological studies (based on reactions as yet unpublished observations on reactions in human beings from a Smadel-Jackson prepared purified and concentrated "vaccine, followed by a series of studies in rabbits and mice which demonstrated classical biphasic fever patterns, induction of endogenous pyrogen, local Swartzman reaction, hypersensitivity to catechole amines (intradermal adrenalin), hypersensitization by RE blockade by thorothrast, classical leukocyte response patterns and liver glycogen depletion patterns in mice, etc.). The level of endotoxic reactivity of the antigen suspension was measured by the Limulus assay and was found to be strongly positive. In standard leukocyte suspensions from non-typhus-immunes, it was found that an antigen dose less than 3 ug/ml dry wt. of R. prowazekii produced a low incidence of "non-specific" reactions in the standard lymphoblast transformation test. An antigen dose of 1 ug/ml dry wt. R. prowazekii was chosen, though in most instances graded doses above and below this level were employed because it was found that the optimal stimulating dose varied somewhat from one typhus immune to another.

This small study includes tests with immune and non-immune human subjects, with the serendipitous observations of one individual in the incubation period of murine typhus infection and of individuals before and after infection with R. mooseri or R. prowazekii. As might be expected from lymphocyte blast transformation studies, the data obtained from a sort of scatter diagram, with some paradoxes and some more positive correlations. The following items, recorded in Tables 6-9, are representative of the findings.

(1) Among non-immunes, by history and antibody determinations, considerable variability in lymphocyte stimulation index was found which overlapped somewhat that obtained with lympocytes from immunes. Whether this is due to inherent variabilities in the test, to immunological cross-reaction, as with ubiquitous Proteus species, to endotoxins, etc. is unknown.

(2) The stimulation indices with typhus-immunes, including subjects with long-standing immunity up to 3 decades, with immunity of intermediate duration (1-5 years) or with immunity of very recent origin, were variable from individual to individual, from unexpected by low to high, were variable from time to time of testing, and gave different responses with graded antigen doses.

(3) One subject,without prior baseline testing, serendipitously was tested 3 days before he developed clinically apparent febrile murine typhus fever and showed an unusually high stimulation index with ET antigens which persisted throughout convalescence. A second person, also infected in the same episode showed a marked rise in stimulation index between baseline determination and determination from shortly after the onset of fever to several months after convalescence from brief, antibiotic treated clinical disease.

Satisfying conclusions are difficult to extract from this study.

(1) It is apparent that "non-specific reactions" of some significant degree can be induced by typhus antigens in lymphocytes from people who have had no reasonable opportunity or exposure to typhus antigens in the past. Uninfected yolk sac does not do this. It is possible that cross-reactions with organisms like Proteus or endotoxin-induced reaction may play a part, although these have not been specifically examined and the dose of particulate R. prowazekii antigen was selected to minimize the endotoxin effect.

(2) In general persons who have been infected with murine or epidemic typhus or who have received the living attenuated E strain typhus vaccine in the past and have had variable exposure to typhus rickettsiae have lymphocytes which are stimulated to variable, but significant, degrees.

(3) During the course of typhus infection, either murine or epidemic, there is an increase in lymphocyte stimulation index. The development of a specifically sensitized population of lymphocytes may occur during the incubation period and precede the onset of clinical disease. This is consistent with our previous findings of the development of positive typhus skin tests 7-10 days after vaccination with the attenuated living E strain typhus vaccine (8).

Table 6. Summary of Lymphocyte Transformation and Serological Studies on Two Groups of Subjects with No Known Previous Experience with Typhus Antigens

Subjects	Stimulant (S.I.)[a]			Serological Titers	
1. Medical Students	1 µg NYS	1 µg ET	5 µg PHA	CF	HA
*AH	<1	1.12	116[b]	2	16
ML	<1	8.8	105	<2	<2
NO	<1	10.6	40	<2	<2
VA	<1	5.0	62	<2	<2
RH	<1	2.0	67	<2	<2
*CP	2	3.0	28	<2	<2
LK	<1	4.0	15	<2	<2
*KH	1	1.1	96	<2	<2
DO	1.1	6.4	65	<2	<2
*GW	<1	1.8	27	<2	<2
Mean (± S.D.)		4.4 ± 3.3	62.1 ± 35		
2. Laboratory Personnel					
*LGF	1.6	2.7	114	<2	16
JS	1.1	2.2	82	<2	16
BM	1.6	2.8	81	<2	<2
*CM	<1	2.6	79	<2	4
DC	1.2	1.9	71	<2	<2
AZ	1.4	1.1	148	<2	<2
AFA	3.7	2.0	159	<2	8
PF	<1	1.4	17.5	<2	2
*AK	2.3	2.9	170	<2	4
Mean (± S.D.)		2.2 ± .6	102 ± 49		
		p<< .01			

a S.I. = Stimulation Index
b Numbers underlined represent S.I. with p <.0005
* Denotes female subjects

Table 7. Summary of Lymphocyte Transformation and Serological Studies on Two Groups of Subjects Presumed Immune by Infection or by Vaccination

Subjects	Years (post disease or vaccination)	Stimulant (S.I.) 1 µg NYS	1 µg ET	5 µg PHA	Serological Titers CF	MA
1. Post-disease						
CLW	27	1.1	2.5[a]	85.6	64	256
AC	2.25	1.2	11	155	8	<2
AW	4	<1	9.8	23		
MB	1.25	3.0	6.5	63.8	32	32
MLC	1.75	1.2	6.0	31	16	<2
2. Post-vaccination						
MJ	~15 (E)	1.1	17	39	<2	16
GS	>30 (K,E)*	4.6	6.3	70.6	<2	16
DE	~15 (E + Live)	—	3.7	114	256	256
JO'K	30 + (K)	2.7 (p=02)	5.2	141	<2	2
WM	~15 (E)	2	7.4	68	8	8
RK	11 (K,E)	<1	4.0	68	<2	4

a Numbers underlined represent S.I. with p <.0005

* E = Attenuated E strain vaccine
K = killed typhus vaccine

Table 8. Lymphocyte Transformation and Serological Studies on Subjects Before and After the Onset of Typhus

Subject	Days Following Onset of Illness	Stimulant (S.I.) 1 µg NYS	1 µg ET	5 µg PHA	Serological Titers CF	MA
SA	-3	<1	4	131	<2	2
	+4	ND	4.8	31		
	+11	<1	18.4	121	16	<2
	+25	ND	33	247	16	<2
	+39	ND	18	ND	16	<2
EO	-30	2.2	2.5	55	<2	4
	+4	1.3	8.7	53	<2	2
	+18	ND	8.9	40	128	4
	+39	ND	12	ND	128	4
BH	+60	<1	3.8	26.5		
	+143	ND	10.3	ND	16	<2

Table 9. Results of Migration Inhibition Studies Using Unfractionated Leucocytes from Donors Varying According to Previous Experience with Typhus Antigens

Subject	Immune Status	M.I. (% of Control)	Ag conc.
BM	"Non Immune"	0.82	75 µg
JS	" "	0.63	"
WM	Vaccine, Live Rugs	0.44	"
MJ	" " "	0.72	50 µg
OE	" " "	0.77	"
AC	Previous Disease	0.61	75 µg
AW	" "	0.75	"
EO	" "	0.73	50 µg

2. Antirickettsial action of stimulated human leukocytes. Several years ago when our work on anti-rickettsial macrophage cytophilic antibodies in the mouse and human system (14) was completed, just before work on macrophages ceased for the time, Beaman introduced lymphocytes from a typhus convalescent human subject into cultures of R. mooseri-infected human macrophages on several occasions. In some, but not all, experiments, there appeared to be inhibition of rickettsial growth. Subsequent work with R. mooseri infection in guinea pigs demonstrated by passive transfer experiments the salient role played by splenic lymphocytes from immune guinea pigs, in contrast to immune serum, in controlling rickettsial growth at the cutaneous site of inoculation in adoptive immunized animals (15-19). Moreover, detailed studies of the progress of R. mooseri infection in the guinea pig, from site of intradermal inoculation through blood to distant internal organs (as spleen and kidney), showed the rickettsial multiplication was controlled in the skin at the site of inoculation within 3-5 days, prior to the detection of serum antibodies. In contrast infection in the spleen was demonstrable only after serum antibodies were detectable, i.e. 7-9 days after inoculation. Rickettsiae in the spleen then underwent rapid multiplication for 2-3 days to attain a peak of 10^3 to 10^4 PFU per spleen before control mechanisms were expressed. This evolution and control of infection in the spleen, in the face of serum antibodies and after control of infection at a distant site (skin), suggested that some stimulus was needed, such as perhaps the build-up of an antigenic mass, before control mechanisms were recruited to, and expressed in, this site. In view of the unique capacity of "immune" lymphocytes to effect control of typhus infection in tissue (as skin), this phenomenon appears to bear some similarities to the antigen-dependent homing phenomenon of lymphoblasts described by Jungi and McGregor (30) for the mouse-Listeria system. Further detailed work, possibly in the more convenient and well-defined mouse system, is required to identify specifically the subpopulations of lymphocytes responsible for controlling typhus rickettsial proliferation in tissues and to study the dynamics of these cells at the sites of infection. Studies somewhat similar to those in the scrub typhus system (20,21) are currently under way simply to establish the fact that generically T-lymphocytes are indeed specifically involved. The dynamics of the cell interactions in developing lesions, the identification of the subpopulations of lymphocytes involved, their state of activation and factors which are concerned with homing of specific cell types into the lesions are subjects for future studies.

Effector mechanisms, however, are amenable to some degree of investigation at the present time. A group of human subjects who had experienced either R. mooseri or R. prowazekii infections, or both, at various times in the past was available as a source of circulating lymphocytes. Methods for collecting and stimulating in vitro peripheral cells for lymphoblast transformation studies were established in the laboratory (see above) and quantitative, reproducible systems for measuring intracellular rickettsial growth had been developed in these laboratories.

We, therefore, took the opportunity to make some preliminary and exploratory observations on the effect of the supernatants of lymphocytes from typhus-immune and non-immune subjects, unstimulated and stimulated with R. prowazekii antigen and PHA, as in the lymphoblast transformation studies,

R. prowazekii-infected cells in our standard in vitro growth system. The results were so striking and potentially important that this matter has dominated our attention for the past few months - hence, the paucity of progress on the basic genetics of R. prowazekii, although as will be seen there may be a clue here to some of the problems of measuring low mutant frequencies in the plaque system. These studies were spurred on by the subsequent publication by Chincilla and Frenkel (27) of the detection of soluble products of immune lymphocytes which have a specific action on intracellular Toxoplasma.

Since typhus infections in Man are characterized by parasitism of endothelial and perhaps some other non-phagocytic somatic cells [we have found every vertebrate cell tested (fish, avian, mammalian) to be capable of supporting the growth of R. prowazekii], rather than by parasitism of macrophages, histocytes and Kupffer cells, as in Coxiella burnetii infection, and hence would expect that perhaps lymphocyte-mediated control of typhus infections to be capable of being expressed in cells which are not professional phagocytes, we chose first WI-38 and then the F-1000 human fibroblast cells and secondary chick embryo fibroblasts as host cells for the rickettsiae in which to examine the effects of lymphocyte supernatants. We wished to examine specifically the action of lymphocyte products on rickettsial infection of tissue cells, not "professional" phagocytes. [Note that we have previously shown that anti-typhus antibody with or without complement, divalent cations, lysozyme is not rickettsiacidal and does not prevent infection of CE cells (12,13), that antibody has a strong opsonizing action for both PMN's and monocytic cells in vitro and in vivo (5,7,9,10,14), that in vitro monocyte-derived human macrophages phagocytize R. prowazekii and R. mooseri but are unable to retain them within phagosomes so that they escape into the cytoplasm, multiply and destroy the macrophage but that antibody on the surface of the rickettsiae but not cytophilic antibodies on the macrophage surface cause retention of the rickettsiae within phagosomes with their subsequent destruction (9,10,14). We have not yet systematically investigated the capacity of human macrophages in various states of non-specific and specific lymphocyte-mediated activation (under investigation now) to dispose of the rickettsiae without antibody. The latter would be in the line of classical CMI and may be the explanation for the observations of Hinrichs and Jarrells (25) on C. burnetii and of Nacy-Mehady, Meltzer and Osterman (22,23) on R. tsutsugamushi infection in macrophages. We believe that macrophages, either through opsonizing antibody or lymphocyte-mediated activation probably play an important role in the defense against typhus, but we also believe that there may be other important lymphocyte mediated mechanisms for control of intracellular infection in cells which are primary targets in the disease and which are not "professional" phagocytes. Such factors might help explain some of the observations from the guinea pig model. Hence, our excitement about the current phenomenon and its relation to the immunological objectives for a good vaccine.]

Owing to tedious nature of the assay procedure, though highly reproducible and quantitative in many respects, though incapable of quantitative, or even qualitative,differentiation between a specific action on intracellular rickettsiae and selective cytotoxicity directed against infected cells at some stage in the growth cycle, progress has not been as rapid as we would have liked. We have not yet been able to test all the pertinent variables that

would be profitable with the present system, much less devise new systems which will accurately measure specific components as their possible participation arises logically from the results of antecedent studies. The probability is high that we are dealing, not with a single factor, but rather with a series of complex factors, some of which have counter parts in other systems. We have not yet even been able to do a systematic study on the optimal conditions for production of large batches of active lymphocyte products due to manpower limitation, etc. Nevertheless, we believe that we have been able to accumulate enough information to establish the fact that we are probably dealing with factors which have apparent specific activity on R. prowazekii-infected cells and that some of these factors can be elicited from blood cells (probably lymphocytes) from both immune and non-immune subjects by stimulation with killed R. prowazekii suspensions and by the T cell mitogen, PHA.

The phenomenon understudy is illustrated by the results illustrated in Figure 4 . When human-derived cells (in this instance WI-38 cells) and chicken embryo fibroblasts were infected in suspension under standard conditions with R. prowazekii (Breinl), distributed to slide chamber cultures and exposed 6 hrs later to (a) medium control, (2) supernatant from unstimulated leukocytes, from a typhus-convalescent subject and (3) supernatant from leukocytes from this subject cultured 24 h in the presence of 1 µg/ml dry wt. formalin-killed R. prowazekii suspension, two patterns emerged. (1) The supernatants from unstimulated lymphocytes had no apparent effect on the either non-infected cells or on rickettsia-infected cells. (2) The supernatants from R. prowazekii-stimulated leukocytes had a dramatic effect on R. prowazekii-infected human cells, but not on R. prowazekii-infected CE cells. Closer examination revealed that, while the ET-stimulated leukocyte supernatant had no apparent effect, by microscopic examination, on uninfected cells, either WI-38 or CE, it had a marked cytotoxic effect (as judged by cell loss) on R. prowazekii-infected human origin WI-38 cells but not on CE cells. The assay system, as represented in the figure, shows an apparent effect also on rickettsial growth. However, this may be an artifact of the assay system because similar results would be expected if the action were confined to cells with a given level of infection. Nevertheless, rickettsiae within surviving infected cells often appear morphologically damaged. Obviously, we cannot at this time discriminate between direct, primary damage of rickettsiae by lymphocyte mediators and secondary effects resulting from unfavorable growth conditions in damaged host cells. Regardless, the phenomenon observed is of great potential importance, worthy of intensive pursuit, as a possible effector mechanism in CMI to typhus infections.

Similar observations were subsequently made on blood cell supernatants from both immune and non-immune subjects, as partially recorded in Table 10. Certain other variables have been introduced, either regularly or as time has permitted. The current status of this study, still in its infancy, can be summarized as follows.

When the leukocytes from the blood of human subjects who are either not immune to typhus or who are immune by virtue of infection to either R. prowazekii or R. mooseri are stimulated in vitro with either 1 µg/ml formalin-killed purified R. prowazekii (Breinl) suspension or the T cell mutogen PHA, soluble factor(s) are produced which have the following properties.

(a) No visible cytotoxic action on human derived cells (WI-38, or F-1000 fibroblasts) or on secondary chicken embryo (CE) fibroblasts).

(b) A dramatic cytotoxic reaction, recognized by significant loss of cells, is specifically directed towards R. prowazekii-infected human cells (WI-38 or F-1000) but not against comparably infected CE cells (Host-cell specificity). Thus, there is a product of lymphocytes, not necessarily specific as to stimulating agent or immune status of lymphocyte donor which has specific action on rickettsia-infected human but not CE cells.

(c) Although the graphs presented suggest a direct anti-rickettsial action, this cannot be evaluated accurately at the present time with the current assay system. An impression of some direct anti-rickettsial action exists but is not proven.

(d) When adherent cells were removed from the leukocyte suspensions from the blood of an immune subject, ET stimulation of the non-adherent cells produced a supernatant fluid which behaved in the same fashion as described above. Though we do not have a good quantitative assay system yet, it is likely that the active product(s) was derived from lymphocytes rather than monocytes or PMN's.

(e) A single experiment with a Karp-like strain of R. tsutsugamushi from Pakistan failed to demonstrate an effect of ET-stimulated lymphocyte supernate as measured by either cytotoxicity on infected human F-1000 cells or in CE cells or inhibition of rickettsial growth in either cell line (? organism specificity).

Though the study is still in its infancy, despite an enormous effort, the prospects are exciting. A putative lymphocyte factor which causes specific selective destruction of R. prowazekii-infected somatic cells, thus exposing the formerly intracellular rickettsiae to the established control mechanism of epsonization by antibody and subsequent destruction within macrophages [we have shown that antibody in the medium has no effect on intracellular rickettsiae (13)] or their destruction in macrophages "activated" by classic CMI mechanisms could be potent mechanisms in the control of rickettsial infection. The further prospect, as yet unproven but certainly possible, of a lymphocyte factor which has a direct antirickettsial action in the cells which are normally the "target cells" in typhus infection is even more exciting.

Obviously, there are many additional studies that are necessary to characterize and sort out the phenomena involved. For example, will a B cell mitogen, such as LPS (which typhus rickettsiae contain) elicit the production of similar or different, "anti-rickettsial" factors? Are these factors produced by T cells, B cells, or both (lymphocyte separation experiments). Is the action solely on typhus-infected cells or (i) is there organismal specificity involved as the single experiment with scrub typhus would suggest or (ii) is there also direct antimicrobial action? We are currently exploring various quantitative measures of cytotoxicity - e.g., dye-exclusion methods, release of tritiated thymidine, ^{51}Cr, and rubidium, each of which has its special merits. We hope to differentiate between

cytostasis and cytotoxicity and are designing experiments to test direct anti-rickettsiae action - probably using the immobilized host cell technique as opposed to the suspended cell technique (Wisseman et al., to be published) because lymphocyte supernatant fluids tend to agglutinate host cells. Are these effects attributable to other previously defined action of lymphocyte supernatants, etc. (interferons, anti-tumor action, lymphotoxin, etc.)? We have the technical capacity to do most of these, including attempts to separate cytotoxic from antimicrobial action by lymphocyte products. Quantity of man-power is the limiting factor.

3. Challenge of an immune subject with virulent R. prowazekii. Because of certain paradoxical and variable results in lymphoblast transformation and production of lymphocyte anti-rickettsial factor, a human subject (CLW) with solid typhus immunity of long standing, received intradermally on the volar surface of the left forearm 10^5 PFU of the plaque purified Breinl strain of R. prowazekii (known to be virulent for man on the basis of laboratory infections) and on the corresponding site on the right forearm with the same quantity of the same y.s. seed of R. prowazekii (Breinl) that had been formalin killed. The inoculum contained an estimated 5-7 x 10^5 viable R. prowazekii - enough probably to infect 10^5 non-immune people. It contained about 0.2 µg dry wt. rickettsial bodies (antigen). As expected, the site of inoculation of the killed organisms produced an intensive reaction characterisitic of the delayed type hypersensitivity previously described by others and by us (8). The reaction at the site of the live challenge was considerably more intense, and larger with actual central necrosis. Significantly, neither epitrochlear nor axillary lymph nodes reacted sufficiently to become palpable. The only systemic reaction was one of mild malaise the second night, comparable to that induced by an influenza vaccination but less than that produced by a standard typhoid vaccination in this subject. The main observations from this challenge experiment were as follows (See table 11). (1) Immunity to typhus established by infection is solid. The major host-parasite interaction takes place at the local level without evidence even of regional lymphatic hypertrophy. (2) The vigor of the interaction with living organisms, as reflected by the size of the response and necrosis, is greater than is given by an equivalent number of formalin-killed organisms through the mechanisms of DTH. (An Arthus reaction cannot be excluded outright, but the absolute quantity of antigen introduced was very low for this.) (3) A marked response, consistent with DTH was elicited by killed organisms. (4) There was no significant change in ET and MT MA antibody titers as a result of this challenge. The relative antibody titers determined by the MA test reflected the dominant influence of the primary infection, as opposed to initial killed vaccine prior to primary infection, to the nature of challenges subsequent to primary infection and to the identity of the challenge organism. (5) There was no change in stimulation index in the lymphocyte blast transformation test with R. prowazekii antigen. (6) There was no dramatic change in the action of supernatants from R. prowazekii or PHA stimulated lymphocytes on R. prowazekii-infected F-1000 human fibroblast cells. Thus, the entire action between host and rickettsiae appeared to be a local phenomenon, determined at the site of inoculation, with very little evidence of systemic response. Despite the fact that some months had intervened between this experiment and the last substantial exposure to R. prowazekii, the episode was merely a local skirmish, in which

DTH and "toxicity" was expressed but no antibody or circulating lymphocyte response was recognized. This is reminiscent of the early work of Castaneda (2, 3) and our recent (16) observations in the guinea pig.

This kind of immunity appears to be qualitatively different from that induced by conventional killed vaccines. This has enormous implications with respect to the mechanisms of immunity to typhus and the objectives for a killed vaccine which induces a comparable state of immunity. It emphasizes the need to define at a basic level very clearly the important mechanisms of immunity to typhus and to design vaccines which induce this kind of immunity. At the present time, only the living attenuated E strain vaccine does this. However, it is not beyond the current state of the art in immunology to prepare a non-replicating vaccine (possibly a component or "subunit" vaccine devoid of the endotoxic reactogenic components that will stimulate the appropriate arm of the immune response, which will be stable on storage (as in a military stock pile) and which will elicit a comparable level of immunity, without the reactogenicity and spectre of possible reversion to virulence as with the current living attenuated E strain vaccine, with its superior benefits and both hypothetical and real disadvantages. There is good reason to believe that the technology of immunology is such that "vaccine" preparations can be made with rickettsial components which will specifically stimulate the mechanisms responsible for real immunity in rickettsial diseases, once these have been defined and thus, the objectives have been identified. (See section on rickettsial proteins:) This has been the ultimate goal of the PI for some years. His concern with the living attenuated E strain vaccine has been that it is the only proven really effective vaccine currently available that might be able to have an impact <u>at this time</u> on the major world-wide typhus problem (as in Africa and South America), despite its problems, both acknowledged and theoretical. For example, recent field experiences in Central and South America have served to underline the role of a single-dose vaccine which induces real protection as the only practical solution to the typhus problem there, as well as in Africa and the highland areas of Asia. From a military point of view, it would be desirable to have a vaccine which is <u>effective</u> and which is stable on storage, so that it can be held in reserve until a problem arises. It is the belief of this investigator that (1) a basic study of the mechanisms of immunity to typhus, (2) a definition of the antigenic components of the rickettsia and (3) a study into the means by which "(2)" can be presented to people to achieve "(1)" will lead to a better anti-typhus vaccine. Until this is achieved, however, it is essential to study the potential hazards of the only typhus vaccine <u>proven</u> to protect against disease - namely, the living attenuated E strain vaccine. (There is no doubt that properly prepared conventional killed vaccines will reduce <u>mortality</u> in infected people but no good quantitative data exist on its capacity to protect against infection. In this post-antibiotic era, what was formerly a "miracle" with respect to the typhus problem - no deaths - is just not good enough. Reliable prevention of disease must be the objective. I stress this very strongly because some advisors, whose experience was in World War II when prevention of death by "vaccine" was a true remarkable advance, still operate under the aura of this limited, but significant success. Things have changed. We can now expect of modern immunology protection from disease, not just protection from death. Antibiotics will do the latter).

Table 10. Host cell specific cytotoxicity against R. prowazekii-infected cells of supernatants from leukocytes from non-immune and immune human subjects stimulated by R. prowazekii or PHA.

Subject	Typhus Immune Status	Mito-gen	Lymph. Stim. Index	LIF % Contr.	"Anti-Rickettsial" Action						Inter-feron Titer
					Human Cells			CE Cells			
					Cytotox.		Apparent	Cytotox.		Apparent	
					N-Inf.	Inf.	Ant-R	N-Inf.	Inf.	Anti-R	
Pat	Non-immune	ET PHA			pending			pending			
Shap.	" "	ET PHA			pending			pending			
M	" "	ET PHA	2.0 26.9	37 43	0 0	3+ 3+	3+ 3+	pending			
Z	" "	ET PHA	<1 12.5	63 99	0 0	2+ 3+	2+ 2+	pending			
T	" "	ET PHA	7.8 19.0	21 79	0 0	3+ 2+	3+ 2+	pending			
CLW (1-5-79)	Immune	ET PHA	8.0 ND	48 ND	0 ND	3+ ND	3+ ND	ND ND	ND ND	ND ND	
AW 10-6-78	"	ET PHA	3.9 58.9	37 92	0 1+	3+ 2+	3+ ND	ND ND	ND ND	ND ND	
EO 4-21-78	"	ET PHA	ND ND	ND ND	0 ND	3+ ND	3+ ND	0 ND	0 ND	0 ND	
EO 10-5-78	"	ET PHA	19.6 67.4	22 101	0 0	2+ 2+	3+ 2+	ND ND	ND ND	ND ND	
BAH 7-15-78	"	ET PHA	ND ND	77 87	0 2+	0 2+	2+ 3+	0 0	0 0	0 0	
AC 6-6-78	"	ET PHA	ND ND	ND ND	0 ND	2+ ND	3+ ND	0 ND	0 ND	0 ND	

Table 11. Antibody and Cellular Responses of Immune Human Subject to Virulent Challenge with R. prowazekii

Day of Challenge	Serum Antibody Titer								Properties of Leukocyte Supernatants						
	MA		CF			IFA							Action on Hu Cells		
	ET	MT	Sol	ET	MT	I_gM	I_gG	I_gA	Mitogen	Lymp. Stim. Index[2]	LIF % Contr.[3]	Interferon Titer[4]	Cytotoxicity[5] N-Inf.	Cytotoxicity[5] Inf.	Anti-R[6]
-2	8	256							-	-	-	-	-	-	-
-1	-	-	-	-	-	-	-	-	ET	9.4	46	-	0	3+	2+
									PHA	37.5	107		0	2+	1+
0	10^5 PFU R. prowazekii (Breinl) i.d.														
+7	8	256							ET	6.5	57	pending	0	3+	3+
									PHA	32.0	101		0	1+	2+
+14	16	512							ET	7.4	63		0	3+	3+
									PHA	5.4	107	-	0	1+	1+
+85	-	-	(large batch)						ET	8.0	48	pending	0	3+	3+
									PHA	ND	ND		ND	ND	ND
+97	16	512							ND	-	-		-	-	-

1. ET = 1 µg/ml formalin-killed, purified R. prowazekii (Breinl)
   PHA = 1 µg/ml phyto hemagglutin
2. Lymphocyte Stimulation Index
3. LIF = leukocyte migration inhibitory factor
4. Interferon titer determined with VSV in F-1000 Hu fibroblasts
5. Cytotoxicity as estimated by visual observation of cell loss: 1+ = 25% loss; 2+ = 50% loss; 3+ = 75% loss. N-Inf = non-infected cells F-1000 Hu fibroblasts; Inf. = R. prowazekii infected F-1000 Hu fibroblasts.
6. Anti-R = apparent antirickettsial action from growth curves. May be artifact from selective loss of infected cells.

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Typhus - our laboratory

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C. Miscellaneous Studies. Because of the presence of interested students, fellows and faculty, it has been possible to explore at minimal cost additional related basic projects on growth and protein composition of R. prowazekii. Two such recent pertinent projects, one completed and the others in progress, are presented below.

1. Differential CO_2 requirement for intracellular growth in cell culture by selected rickettsiae (with Anna Kopmans). HEPES - buffered Dulbecco's media were developed which were shown by preliminary experiments to maintain the medium at pH 7.2 under growth conditions in an air (no added CO_2) and a 5% CO_2 in air atmosphere, and to support the normal growth of unirradiated uninfected secondary CE cells in either atmosphere. Then, using our standard slide chamber culture system with irradiated secondary CE cells, we investigated the requirement for CO_2 in the atmosphere for intracellular growth of the following rickettsial strains:

1. Typhus Group

   a. Rickettsia prowazekii
      (1) the attenuated E strain of long egg passage.
      (2) a recent isolate of low egg passage from a typhus patient in Burundi
   b. Rickettsia mooseri (R. typhi). The egg-adapted Wilmington strain.

2. Spotted Fever Group: Rickettsia rickettsii (Shiela Smith strain), an egg-adapted laboratory strain.

3. Scrub Typhus Group.

   a. Rickettsia tsutsugamushi, Gilliam strain, an egg-adapted laboratory strain.
   b. Rickettsia tsutsugamushi, Sialkot strain (Karp serotype), a "recent" isolate of low egg passage.

The salient findings of this study were as follows:

1. In the presence of 5% CO_2 in the atmosphere, all rickettsial strains grew as well in culture with the HEPES-buffered medium as they did in cultures with the conventional bicarbonate-buffered medium.

2. Both strains of R. tsutsugamushi, representing two major serotypes and high and low egg-passage, grew as well in cultures with HEPES-buffered medium in the absence of added CO_2 in the atmosphere as they did in the presence of CO_2.

3. In contrast, all strains tested of the typhus group required added CO_2 in the atmosphere for intracellular growth. Careful examination of the growth curves indicated that, in the absence of CO_2, the organisms underwent an average of less than 1 division over the first 24-48 h of intracellular residence and then failed to multiply further, even though they remained detectable by standard staining (Giménez) procedures for the 48-72 h of observation. However, if the cultures were then placed in a 5% CO_2 atmosphere, growth resumed at a normal rate after a short lag period.

4. The single representative of the spotted fever group. R. rickettsii (Shiela Smith) tested showed a dependence on a CO_2-enriched atmosphere for intracellular growth similar to that of the typhus group.

Thus, different groups currently included in the genus Rickettsia display 2 patterns with respect to the requirement for a CO_2-enriched atmosphere for intracellular growth in cell culture:

(1) Members of the typhus and spotted fever group appear to require a CO_2-enriched atmosphere.

(2) At least 2 serotypes of R. tsutsugamushi (scrub typhus group) do not require a CO_2-enriched atmosphere.

It is interesting that Rochalimaea quintana, formerly included in the genus Rickettsia but removed because it is not an obligate intracellular parasite and can be grown on artificial cell-free medium, also requires a CO_2-enriched atmosphere for growth. These findings have both basic biological and practical value.

2. Proteins of Rickettsia prowazekii (with Ed Oaks and Jonathan Smith).

Separation of a mixture of proteins by a one dimensional technique such as polyacrylamide gel electrophoresis or isoelectric focusing is usually based on a single physical characteristic such as molecular weight or isoelectric point. Whole cell digests contain many polypeptides which have similar molecular weights or isoelectric points and will not be separated in a one-dimensional system. By combining two different electrophoretic separation procedures, more information can be obtained than by either technique in a single dimension.

Recent papers have separated rickettsial proteins by one dimensional techniques (1, 2, 3, 4). Approximately 25-30 proteins of rickettsial organisms have been resolved. Although differences in species were clearly evident by these techniques, the total rickettsial repetoire of proteins has hardly been uncovered since the genome size is approximately 1.5×10^6 or nucleotide pairs (5) or about 1×10^9 daltons m.w. (see above) - i.e., about 40% of that of E. coli. We have combined preparative isoelectric focusing with polyacrylamide gel electrophoresis, as O'Farrell did with E. coli (6), in an attempt to resolve the complete protein complement of rickettsiae

a. Two-dimensional electrophoresis of Rickettsia prowazekii polypeptides.

Renograffin purified R. prowazekii (Breinl), approximately 25 mg dry weight, were prepared for preparative isoelectric focusing by digesting the intact organisms in a solution of 2% Triton X-100, 6 M urea, and 6 uM PMSF, at 4°C. The solution "cleared" after 45 minutes. Ampholytes (pH 3-11) were added to the mixture of solubilized rickettsiae before pouring the flatbed gel. The proteins were separated on a pH gradient of 3-11, with equilibrium being reached after 15 hours. Fractions corresponding to each pH unit were pooled, eluted from the sephadex bed, dialyzed and concentrated.

The samples were then boiled in electrophoresis buffer (SDS and mercapto ethanol) and electrophoresed in an SDS-polyacrylamide gel. The proteins were then stained with Coomassie blue.

Results:

Each fraction corresponding to a different pH range had clear, distinct polypeptides that were unique to that fraction. The total number of unique polypeptides visible is approximately 100. There were, however, some polypeptide species that appeared in many sequential fractions. These proteins might represent different "isoenzymes" or alternatively a problem attributed to either "mixed micelles" or protein precipatates. The sensitivity of this two-dimensional procedure would be greatly increased by using labeled rickettsial proteins. Preparative isoelectric focusing allows the isolation of large quantities of rickettsial proteins under non-denaturing conditions. The use of the two-dimensional system should allow the determination of subtle differences between species or strains of the rickettsiae.

b. Specific release of rickettsial proteins by various non-ionic detergents.

The solubilization of rickettsial membrane proteins under non-denaturing conditions is necessary for a functional characterization of the isolated proteins. Non-ionic detergents have this solubilizing capacity.

The solubilizing capacity of three non-ionic detergents (Tutor X-100, octyl glucoside, and zwittergent) were determined on Renograffin purified R. prowazekii.

Procedure:

1.5 mg (dry weight) of R. prowazekii (Breinl) was dissolved in a phosphate buffered detergent solution. After digestion, an aliquot of each sample was looked at by negative stain. After centrifugation, the pellets were dissolved in SDS and B-mercaptoethanol and then electrophoresed in SDS-polyacrylamide gels.

Results:

The detergent-treated rickettsiae were still intact as viewed by negative stain. However, when the protein profile of the pellets were monitored by PAGE, marked differences in the detergents were noted. Zwittergent had the best solubilizing capacity. Three major polypeptides and many minor ones were completely removed. Octyl glucoside removed two major proteins, and Triton X-100 solubilized only one major protein along with some additional minor species.

The solubilizing properties of these detergents will allow the specific removal of polypeptides from rickettsial cells and will simplify purification schemes of these major proteins.

c. Comparisons of Heavy and Light Band R. prowazekii by SDS-PAGE.

Renografin purified rickettsiae corresponding to the heavy and light bands on renografin gradients were digested in SDS-mercaptoethanol, and electrophoresed on SDS-polyacrylamide gels.

Results:

The heavy and light band rickettsiae have only minor differences (1-2 minor bands) in their protein profiles as determined by Coomassie blue staining.

d. We have developed analytical and preparative techniques that are superior to the published techniques used to study rickettsial proteins.

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Figure 6. Protein profiles (slab SDS-PAGE) of Light (L) and Heavy (H) bands of *R. prowazekii* (Breinl) from Renografin gradient.

Renografin Gradient Purified

R. prowazekii (Breinl)

H L

d: 1.15 1.13

Figure 7. Protein profiles (slab SDS-PAGE) of whole Rickettsia prowazekii (Breinl) and various detergent extracts.

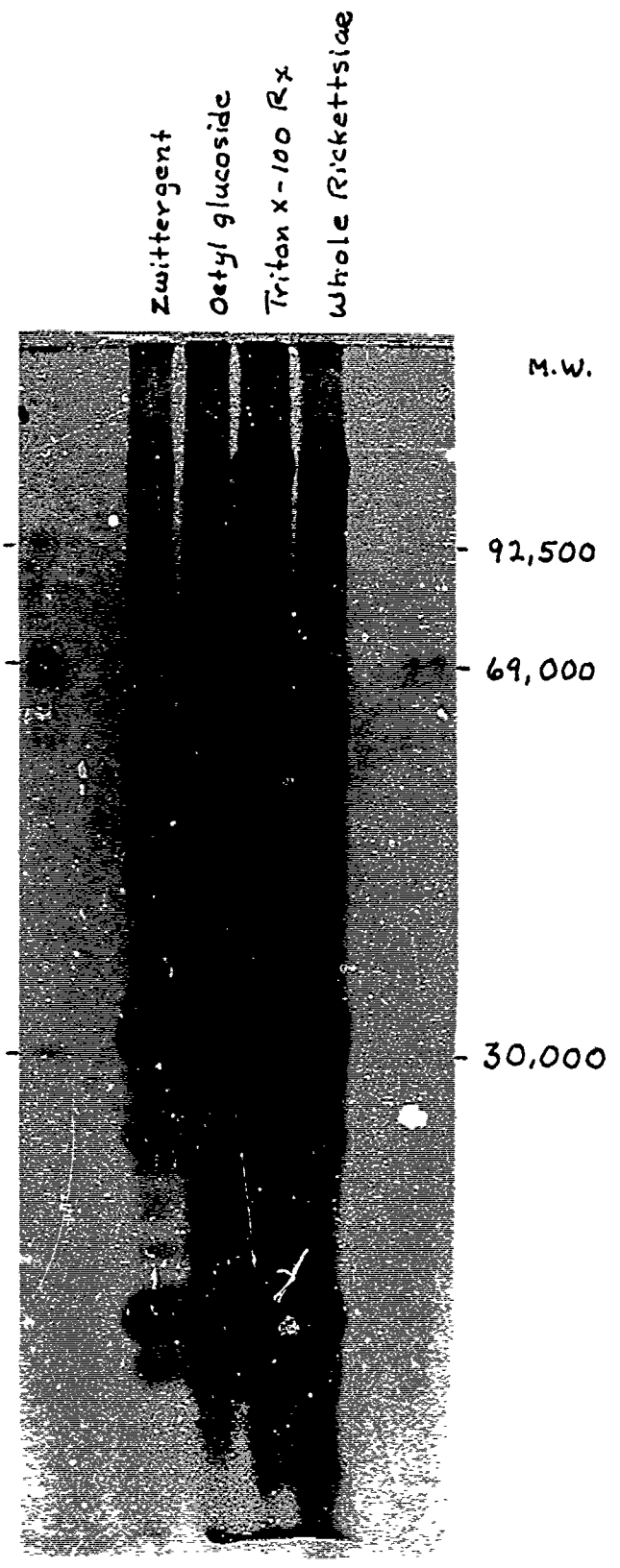
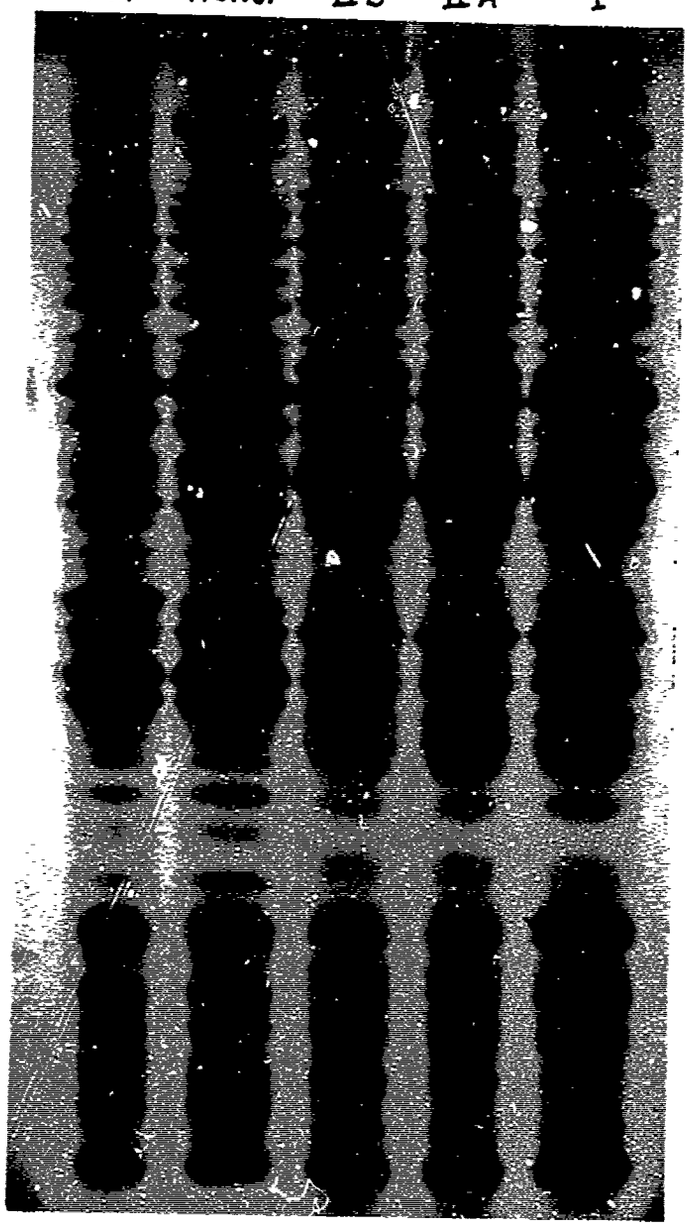

Zwittergent
Octyl glucoside
Triton x-100 Rx
Whole Rickettsiae
M.W.
92,500
69,000
30,000

Figure 8. Protein profiles of Rochalimaea quintana and Baker's Vole Agent. (slab SDS-PAGE) Note the similarity between the Fuller and Heliodoro strains of R. quintana and between the 3 lines of Baker's Vole Agent, but the marked differences between R. quintana and Baker's Vole Agent.

R. quintana Baker's Vole Agent

Ful. Helio. IIB IIA I



II. STUDIES ON THE SPOTTED FEVER GROUP OF RICKETTSIAE

The main objectives of this portion of the contract, which has not been high a priority as the studies on typhus with respect to allocation of man-power and resources for practical and operational reasons, are (1) to devise methods and principles by which spotted fever group rickettsiae can reliably and easily be identified into biologically and medically discrete sub-groups or species (a sordid state of affairs today) and (2) to identify by such means the array of spotted fever group rickettsiae which we have isolated in Pakistan and others have isolated in Thailand, Israel and Czechoslovakia.

Two methods will be employed to perform these studies: (1) serological (we have some substantial preliminary evidence that the MA test employing mouse immune ascitic fluid will d scriminate well between established species but other serological tests, such as the IFA as recently employed at the Rocky Mountain Laboratory will also be employed if necessary) and (2) DNA homology studies, an apparently powerful tool as described in "A" above.

The efforts in the past year have been devoted to the following:

1. Production from L-cell seeds of 12-14 spotted fever group strains, both established and recent isolates of mouse immune ascitic fluid. The production has been completed, pending systematic testing.

2. Production in eggs of sufficient rickettsial material from each of the strains under study to (a) provide enough MA antigen to perform the serological tests and (b) provide enough DNA to perform the homology studies. Owing to the low yields of these organisms in eggs, compared with typhus group agents, this has been an extremely slow and laborious procedure. It takes an average between 6 and 8 weeks of production per strain to accumulate enough material for these purposes. We have made substantial progress, but project now that it will be mid-to-late summer 1979 before all the necessary material will be on hand to begin the definitive work.

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